Trials in Vaccinology 3 (2014) 81–88

Contents lists available at ScienceDirect

Trials in Vaccinology

journal homepage: www.elsevier.com/locate/trivac

A novel dual promoter DNA vaccine induces CD8$^+$ response against *Toxoplasma gondii* sporozoite specific surface protein "SporoSAG" through non-apoptotic cells

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ARTICLE INFO

Article history:
Received 29 December 2013
Revised 9 April 2014
Accepted 16 April 2014

Keywords:
Toxoplasma gondii
DNA vaccine
SporoSAG
Bcl-xL *anti*-apoptotic protein

ABSTRACT

Toxoplasma gondii is a protozoan parasite that can infect all warm blooded animals including humans. During natural course of *T. gondii* infection, bradyzoites or sporozoites invade the intestinal cells and turn into tachyzoite form in 12–18 h. Therefore, a vaccine against toxoplasmosis is required to induce protective immune response initially in intestines against bradyzoites or sporozoites. The present study aimed to generate a DNA vaccine containing a sporozoite specific surface antigen "SporoSAG". To increase antigen specific-CD8$^+$ T lymphocyte response, *anti*-apoptotic Bcl-xL gene was inserted to vaccine as a molecular adjuvant.

For the construction of DNA vaccine, SporoSAG gene was inserted after CMV promoter and Bcl-xL gene was inserted in frame with EGFP after IRES promoter (pSporoSAG/Bcl-xL). Bcl-xL expression and functionality as well as humoral and cellular immune responses were demonstrated by Western blotting and flow cytometer.

Western blotting and flow cytometer analyses rationalized Bcl-xL expression that impedes apoptotic cell death. The ratio of pSporoSAG/Bcl-xL transfected cells were significantly higher than empty pEGFP control plasmid ($P < 0.05$). Analysis of sera obtained from vaccinated mice showed strong *anti*-SporoSAG specific IgG response. The ratio of CD8$^+$ T lymphocytes secreting IFN-γ significantly increased compared to controls ($P < 0.0001$), indicative of protection against toxoplasmosis.

The results of this study reveal the ability of SporoSAG protein to induce CD8$^+$ T lymphocyte response for the first time. Overall, SporoSAG protein can be included to multivalant vaccine formulations in future studies to increase the protection in infections acquired through *T. gondii* oocysts.

Introduction

Toxoplasma gondii is an obligate intracellular parasite infecting all warm-blooded animals, including humans. *T. gondii* infection causes congenital toxoplasmosis in newborns that may lead to fetal anomalies, retinochoroiditis leading to blindness, lethal toxoplasmic encephalitis in immune compromised patients, and organ failure in transplantation patients. Currently, toxoplasmosis is linked to schizophrenia and other mental disorders [1,2].

Worldwide, 500 million people are estimated to be infected with *T. gondii*. Infection rates among pregnant women range from 37% to 58% in Europe, 10.8% in USA, and 30 to 60% in Turkey [3–6]. In United States, 400–4000 infants are estimated to born with congenital toxoplasmosis each year and up to 1,26 million cases of ocular toxoplasmosis are detected [6,7].

The present drugs are not 100% effective against toxoplasmosis and thus, a vaccine appears as an alternative in protection against *T. gondii* infections. Finally, after classification of *T. gondii* oocysts in category B bioterrorism agents as a water safety threat, the demand for a protective vaccine against toxoplasmosis has increased [8]. This remark was supported by tainted water outbreaks such as the British Columbia, Great Victoria outbreak (affected 7718 people), Santa Isabel do Ivai outbreak (located in

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http://dx.doi.org/10.1016/j.trivac.2014.04.003
1879-4378/

South Brazil that affected hundreds of people) and recently İzmir outbreak (affected 171 Air force academy recruits) [6,9,10].

T. gondii is acquired naturally through ingestion of tissue cysts (contains bradyzoites) or oocysts (contains sporozoites). After the ingestion, released sporozoites and bradyzoites invade the intestinal cells and turn into tachyzoites in 12 and 18 h, respectively [11,12]. To date, vaccine candidate antigens were almost always selected randomly and mainly targeted to induce protective immunity against the tachyzoite form of the parasite. Tachyzoites invade host cells abruptly and immediately embed themselves in a protective parasitophorous vacuole (PV) in the host cell. In the PV, tachyzoites retain themselves from the host immune response. Almost all of the vaccination strategies against the rapid tachyzoite invasion form induced inadequate immune response to initiate clinical studies. A preferred vaccine against toxoplasmosis has to induce protective immune response against bradyzoites or sporozoites during the initiation of infection as well as tachyzoites thereafter.

Protective immunity against *T. gondii* is achieved by Th1 responses. Therefore targeting MHC-I restricted antigens to induce $CD8^+$ T lymphocyte response is the sole aim of vaccine development strategies [2,13]. The present study aimed to generate a DNA vaccine containing a sporozoite specific surface antigen "SporoSAG" for the first time, to block the sporozoites as they are released from the oocysts in the intestine [14]. To increase the efficacy of the DNA vaccine, *anti*-apoptotic Bcl-xL (B-cell lymphoma-extra large) gene was inserted to the dual expression vaccine plasmid as a molecular adjuvant to increase the antigen specific-$CD8^+$ T lymphocyte response.

Materials and methods

DNA vaccine constructs

DNA vaccines were constructed using a dual promoter pIRES2EGFP vector (Clontech, USA). The vaccines designed for this study were pBcl-xL; encoding the Bcl-xL *anti*-apoptotic protein gene (GenBank no: AAC53459.1), pSporoSAG; encoding SporoSAG (GenBank no: AY492338) and pSporoSAG/Bcl-xL; encoding both SporoSAG and Bcl-xL.

pBcl-xL plasmid was constructed as described [15] and the final pBcl-xL plasmid encoding Bcl-xL gene in frame with the EGFP (Enhanced Green Fluorescent Protein) is under the control of the IRES promoter. SporoSAG gene was isolated from pET28a/SporoSAG plasmid by the following primers (SporoSAG forward: 5′-AGATCTATGGGCAGCCATCATCATC-3′ and SporoSAG reverse: 5′-AGATCTTCAGTGGTGGTGGTGGTGC-3′) containing the *Bgl*II restriction site to be cloned within the unique BglII site of the pIRE2EGFP. The pSporoSAG and the pSporoSAG/Bcl-xL plasmids express the SporoSAG protein under the control of the CMV (Cytomegalovirus) promoter.

To facilitate the excision of the amplicons with the corresponding restriction enzymes, cloning of all intermediate Polymerase Chain Reaction (PCR) products and their subsequent clonings in the final plasmids were performed with the pGEMT-Easy plasmid kit (Promega, USA) according to the manufacturer's protocol. The sequences were confirmed by automatic sequencing using the ABI Prism Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, USA) and the BioEdit genetic analyzer (Ibis Biosciences, USA).

In vitro transfection of Baby Hamster Kidney (BHK) 21 cells and Western blotting

Baby Hamster Kidney (BHK) 21 cells (ATCC No: CCL-10) are transfected with pBcl-xL, pSporoSAG, pSporoSAG/Bcl-xL and empty pEGFP plasmids in 10 cm diameter culture dishes using the Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's protocol. After 48 h, *in vitro* transfection efficacies were observed by EGFP expression using fluorescence microscopy. Next the cells were harvested to evaluate the *in vitro* expression of plasmids encoding Bcl-xL protein by Western blotting.

BHK-21 cells were scraped from 10 cm-diameter culture dishes and counted with a hemocytometer. Equal amount of transfected BHK-21 cells (approximately 5×10^5 cell per each sample) were washed thrice with 1 × PBS (Phosphate buffered saline, pH: 7.4) followed by several cycles of freezing and thawing. Then lysed samples were separated by 12% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE). The separated proteins were transferred to a polyvinylidene difluoride (PVDF) transfer membrane (Immobilon-P, Millipore, USA). Thereafter, the membranes were probed with a 1:1000 dilution of the monoclonal *anti*-Bcl-xL Mab (Santa Cruz Biotech, USA) and next, the membranes were probed with a 1:1000 dilution of alkaline phosphatase-conjugated goat *anti*-mouse IgG (H+L) antibody (Bio-Rad, USA). The blot was developed in diethanolamine buffer (10% Diethanolamine, 0.5 mM $MgCl_2 \cdot 6H_2O$, pH: 9.8) containing 4.3% 5-bromo-4-chloro-3-indolyl phosphate (diluted in dimethylacetamide), 4.1% Nitro-BT (diluted in 70% (v/v) dimethylformamide) (Applichem, Germany) [15,16].

Determining the cell survival effect of Bcl-xL protein

To determine the cell survival effect of Bcl-xL protein, BHK-21 cells were serum deprived 48 h after transfection with pBcl-xL, pSporoSAG, pSporoSAG/Bcl-xL and empty pEGFP plasmids as described [15,17]. Briefly, BHK-21 cells were transfected with 2 μg of DNA vaccine plasmids using Lipofectamine 2000 (Invitrogen) for 4 h using OPTIMEM (Invitrogen). Next, cells were cultured in 10% FBS (Fetal Bovine Serum) supplemented GMEM (Biochrome, Germany) overnight. Then, the cells were washed thrice and maintained for an additional 48 extra hours in media without FBS. Finally, cells were harvested, washed thrice and fixed with Cytofix/Cytoperm solution (Becton Dickinson Biosciences, USA). Thereafter, cells were washed thrice with Perm/Wash buffer and resuspended in Ca^{+2}–Mg^{+2} free PBS containing 1% inactive FCS (Fetal Calf Serum), 0.09% (w/v) sodium azide (pH: 7.4). Cell survival was determined by measuring EGFP in transfected cells using flow cytometer (FACSAria; BD Biosciences).

Animals and vaccination

Female 6–8 week-old Swiss outbred mice, obtained from Ege University Experimental Animal Production Facility, were housed and fed under standard, suitable conformist conditions. The experimental plan was performed under the instructions and approval of the Institutional Animal Care and Use Committee of Ege University for animal ethical norms (Approval no: 2007-42).

Four groups of mice (four per each group) were vaccinated thrice at three week intervals with pBcl-xL, pSporoSAG, pSporoSAG/Bcl-xL plasmids and empty pEGFP as negative control (Table 1). The plasmids were purified using an endotoxin-free plasmid purification kit (EndoFree, Qiagen, USA). During DNA vaccinations 100 μg of plasmids was injected into both tibialis anterior muscle of anesthetized mice. Tail bleeds were performed 3 weeks after each vaccination for the detection of *anti*-SporoSAG specific IgG antibodies.

Cloning of SporoSAG gene into bacterial expression vector

T7 promoter containing pET28a expression vector (10 μg; 5.3 kb; kanamycin resistant; Novagen, USA.) was linearized by BamHI as described [16]. The following primers were designed to

Table 1
DNA vaccine and control plasmid doses and routes of administration used during vaccination of mice.

DNA vaccine groups		Dose and route of administration		
		1st dose (day 0)	2nd dose (day 21)	3rd dose (day 42)
1	Empty pGFP	100 μg (i.m.)	100 μg (i.m.)	100 μg (i.m.)
2	pBcl-xL	100 μg (i.m.)	100 μg (i.m.)	100 μg (i.m.)
3	pSporoSAG	100 μg (i.m.)	100 μg (i.m.)	100 μg (i.m.)
4	pSporoSAG/Bcl-xL	100 μg (i.m.)	100 μg (i.m.)	100 μg (i.m.)

generate a linear acceptor vector with sequences suitable for cloning by homologous recombination, 5′-GTCGACAAGCTTGCGGCCGC-ACTCGAGCACCAC-3′ (forward primer, 33 nt) and 5′-CAGCAAATG GGTCGCGGATCCGAATTCGAGCTC-3′ (reverse primer, 33 nt). The linear acceptor vector was amplified and used during *in vivo* recombination cloning.

PCR amplification of SporoSAG gene

The backbone of SporoSAG (GenBank no: AY492338) gene was kindly obtained from Prof. Michael White. Primers designed to incorporate adapter termini into the PCR product to facilitate directional cloning by homologous recombination ('HiRec') into the pET28a vector. The primers for SporoSAG were 5′-CAGCAAATG GGTCGCGGATCCGAATTCGAGCTCTCGCTTCTTAGC CGAGTAGC-3′ (forward primer, 53 nt) 3′-CAGGGTTGGTGTACATGGCTGTCGACAA GCTTG CGGCCGCACTCGAGCACCAC-5′ (reverse primer, 53 nt). SporoSAG gene was isolated from backbone DNA (1–10 ng) using the designed primers (0.5 μM each), 2 U of TaqDNA polymerase (Fermentas, Germany), 150 μM dNTPs and 1 × TaqDNA polymerase reaction buffer with the following calculated PCR protocol; 5 min initial denaturation step at 95 °C, followed by 30 cycles of 0.5 min at 95 °C, 0.5 min at 50 °C, and 3.5 min at 72 °C, and a final extension of 10 min at 72 °C. The PCR products were visualized by agarose gel electrophoresis, purified using a PCR purification kit (Qiagen, USA) and quantitated by spectrophotometry.

In vivo recombination cloning method

In vivo recombination cloning was performed as described [16]. Briefly, linear pET28a vector, purified PCR product and DH5α cells (Invitrogen) were mixed and heat shocked. Plasmids from overnight culture were purified using plasmid purification kit according to the manufacturer's protocol (Qiagen), visualized by agarose gel electrophoresis and sequenced. The resulting plasmid containing SporoSAG was named pET28a/SporoSAG.

Protein expression and purification

Escherichia coli BL21 (DE3) chemically competent cells (Invitrogen) were transformed with pET28a/SporoSAG plasmid and grown in 1 L LB with vigorous shaking at 37 °C up to an optical density of 0.4, calculated at 600 nm. Then, the cells were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM with vigorous shaking at 37 °C for 4 h. The cells were centrifuged at 5000×*g* and the pellet was resuspended with prechilled loading buffer (50 mM Tris–Cl, pH: 7.5, 0.3 M NaCl). Next, the cells were disrupted with an M-110L microfluidizer processor (Microfluidics, USA) at low temperature under internal pressure of 18,000 psi. The processed sample was centrifuged at 30,000×*g* for ½ h at 4 °C and clarified supernatant was filtered through a 0.45 μm filter (Corning, USA).

Purification of the protein was performed by ÄKTA FPLC, a liquid chromatography system, which is controlled by UNICORN™ software, using a 5 ml HiTrap Chelating HP column (GE Health, USA). Clarified filtered supernatant was applied to the HiTrap column with loading buffer. Then, the column was washed with 150 mM imidazole containing 50 mM Tris–Cl, pH 7.5, 0.3 M NaCl buffer. The recombinant SporoSAG (rSporoSAG) protein was eluted by raising the imidazole concentration to 300 mM. The protein fractions were detected by UV280, confirmed by 12% SDS–PAGE, pooled, and concentrated with Vivaspin filter unit (Sartorius, Germany) at 4 °C.

SDS–PAGE and Western blotting of rSporoSAG

Purified rSporoSAG protein separated by 12% SDS–PAGE was transferred to PVDF transfer membrane and blocked by 0.25% casein containing 1× TBS-T buffer (Tris buffered saline containing Tween 20; 20 mM Tris–Cl pH: 7.8, 0.5 M NaCl, 0.5% Tween 20) for 30 min at room temperature. The membranes were probed with a 1:50 dilution of monoclonal *anti*-polyhistidine antibody (Sigma, Germany) for 1.5 h. Next, the membranes were probed with a 1:2500 dilution of alkaline phosphatase-conjugated goat *anti*-mouse IgG (H+L) antibody (Sigma). Thereafter the blot was developed as described above.

Detection of humoral immune response using Western blotting

To detect the *anti*-SporoSAG specific IgG antibody response, rSporoSAG protein was transferred to PVDF as described above. The membranes were incubated with a 1:100 dilution of vaccinated mouse sera for 1.5 h. Next, the membranes were incubated with a 1:2500 dilution of alkaline phosphatase-conjugated goat *anti*-mouse IgG (H+L) antibody (Sigma). Thereafter, the blot was formed as described above to visualize the immune complexes formed between rSporoSAG protein and *anti*-SporoSAG IgG antibodies in the vaccinated mice sera.

Detection of cellular immune response

To determine the specific cellular immune response elicited by each vaccine, mice were sacrificed three weeks after the third immunization and their spleens were removed. Single cell suspensions of splenocytes were prepared as described [16]. Aliquots of 5×10^5 viable splenocytes were added to each well of 96 well round bottom plate (Nunc, USA). Before stimulation of splenocytes, purified rSporoSAG protein was incubated with polymyxin B to a final concentration of 50 μg/ml for ½ h. Cells were stimulated with 12.5–25 μg/ml rSporoSAG protein for 72 h at 37 °C and 5% CO_2. As positive control, splenocytes were incubated with concanavalin A (Sigma) at a final concentration of 10 μg/ml. As a negative control, only growth medium was used.

During the last 4 h of incubation, monensin belonging to Cytofix/Cytoperm kit (BD Biosciences) was added to each well at a final concentration of 2 μM to allow the intracellular accumulation of cytokines according to the manufacturer's protocol.

T lymphocyte populations were surface stained with Alexa flour 647 conjugated rat *anti*-mouse CD3 (Biolegend, USA), FITC conjugated rat *anti*-mouse CD4 (BD Biosciences), or FITC conjugated rat *anti*-mouse CD8a (Abcam, USA), permeabilized with Cytofix/Cytoperm and labeled with an PE conjugated rat *anti*-mouse IFN-γ (Interferon-gamma) (BD Biosciences) or PE conjugated rat *anti*-

mouse IL-4 (Interleukin-4) antibodies (BD Biosciences) according to the manufacturer's protocol.

Antibodies were diluted in Ca^{+2}–Mg^{+2} free PBS with 1% inactive FCS, 0.09% (w/v) sodium azide (pH7.4) for surface staining and in Perm/Wash solution (BD Biosciences) for intracellular staining. All antibodies were used at a final concentration of 0.5 μg/10^6 cells. T lymphocyte populations, gated in the flow cytometer by $CD3^+$ positive expression, were analyzed to quantify: the percentage of rSporoSAG protein-specific $CD8^+$ T lymphocytes secreting IFN-γ and $CD4^+$ T lymphocytes that expressed IL-4.

Statistical analysis

Data obtained during the study were processed using Prism 5 (GraphPad, USA). A two-tailed unpaired t-test or one-way analysis of variance with 95% confidence interval was used to determine the significance between the vaccination groups. Results of cellular immune response and BHK-21 cells transfection efficacy were expressed as mean ± standard deviation (S.D.).

Results

In vitro transfection

48 h after *in vitro* transfection, EGFP expression was determined using fluorescence microscopy (Fig. 1A). *In vitro* cell survival effect of Bcl-xL inserted plasmids were determined under serum deprived conditions. The percentage of surviving transfected-cells was followed by detecting the plasmid encoded EGFP positive cells in a flow cytometer. The ratio of pSporoSAG and pSporoSAG/Bcl-xL transfected cells were significantly higher than empty EGFP control plasmid ($P < 0.05$; designated by "$*$" in Fig. 1B). In addition, the ratio of pBcl-xL plasmid transfected cells was higher than its counterpart empty pEGFP which does not encode the Bcl-xL protein. Moreover, the percentage of pSporoSAG/Bcl-xL plasmid transfected cells was higher than that of pBcl-xL plasmid (Fig. 1B). It was shown that Bcl-xL protected cells from serum deprived apoptosis.

BHK-21 cells transfected with empty pEGFP, pBcl-xL, pSporoSAG and pSporoSAG/Bcl-xL plasmids were harvested after 48 h of incubation and analyzed by Western blotting to determine the correct expression of Bcl-xL *anti*-apoptotic protein. The *anti*-Bcl-xL monoclonal antibody detected the 26.6 kDa housekeeping Bcl-xL gene encoded-protein (Fig. 2; lanes 1–4); and an additional band of ~54 kDa was also shown in cells transfected with either pBcl-xL or pSporoSAG/Bcl-xL plasmids corresponding to the fusion of the EGFP and Bcl-xL proteins (Fig. 2; lanes 2 and 4).

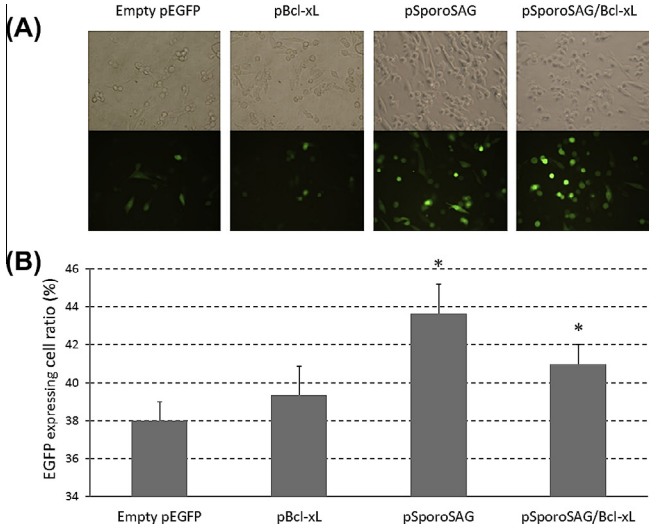

Fig. 1. Determination of *in vitro* transfection efficiency by fluorescence microscopy and flow cytometer. (A) EGFP expressing BHK-21 cells shown in bottom lane (B) EGFP expressing BHK-21 cell ratios transfected with pEGFP, pBcl-xL, pSporoSAG and pSporoSAG/Bcl-xL plasmids under serum deprived conditions.

Anti-SporoSAG humoral immune response

The calculated molecular mass of the rSporoSAG protein, expressed from the 1035 bp ORF, is 36.06 kDa which is detected by monoclonal *anti*-polyhistidine antibody (Fig. 3; lane 1). To determine the humoral immune response, rSporoSAg protein was probed with a pool of serum samples obtained from each group of vaccinated Swiss mice. The mice groups vaccinated with pSporoSAG and pSporoSAG/Bcl-xL induced a strong IgG response against a 36 kDa protein as detected by Western blotting (Fig. 3; lanes 4 and 5). No antibody response was detected in the control groups, in which mice were vaccinated with empty pEGFP and pBcl-xL (Fig. 3; lanes 2 and 3).

Cell-mediated immune response to rSporoSAG expressing DNA vaccines

Splenocytes from individual immunized mice were stimulated *in vitro* with 12.5 μg/ml purified rSporoSAG protein. Flow cytometer analysis was used to determine the ratio of $CD4^+$ and $CD8^+$ T lymphocytes excreting IFN-γ and IL-4 achived by the rSporoSAG expressing DNA vaccines. Vaccine induced IFN-γ and IL-4 secretions are indicators of Th1 and Th2 like responses, respectively. Protection against *T. gondii* is primarily achieved by $CD8^+$ T lymphocytes excreting IFN-γ [2,13].

The $CD4^+$/$CD8^+$ cell ratios of all vaccination groups were shown in Fig. 4A. In this analysis, all vaccination groups were significantly higher than empty pEGFP control ($P < 0.01$; designated by "$**$" in Fig. 4A). The ratio of $CD8^+$ T lymphocytes excreting IFN-γ of all vaccination groups were significantly higher than empty pEGFP control ($P < 0.001$; designated by "$***$" in Fig. 4B). In addition, the ratio of $CD8^+$ T lymphocytes excreting IFN-γ in pSporoSAG/Bcl-xL vaccinated group was significantly higher compared to pSporoSAG vaccinated control group ($P < 0.0001$). The ratio of pSporoSAG control group was significantly higher compared to control group mice vaccinated with empty pEGFP ($P = 0.0005$). The ratio of $CD8^+$ T lymphocytes excreting IFN-γ in pSporoSAG/Bcl-xL vaccinated group was significantly higher than pSporoSAG ($P < 0.0001$) and pBcl-xL ($P < 0.05$) control groups. The ratio of $CD8^+$ T lymphocytes

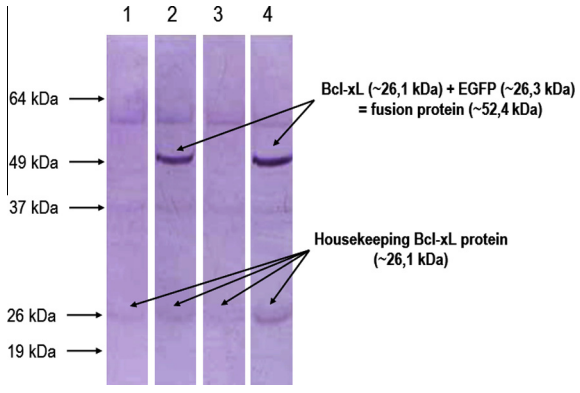

Fig. 2. Determination of *in vitro* transfection efficiency using Western blotting: Detection of Bcl-xL expression using protein lysates obtained from cells transfected with empty pEGFP (lane 1), pBcl-xL (lane 2), pSporoSAG (lane 3), pSporoSAG/Bcl-xL (lane 4).

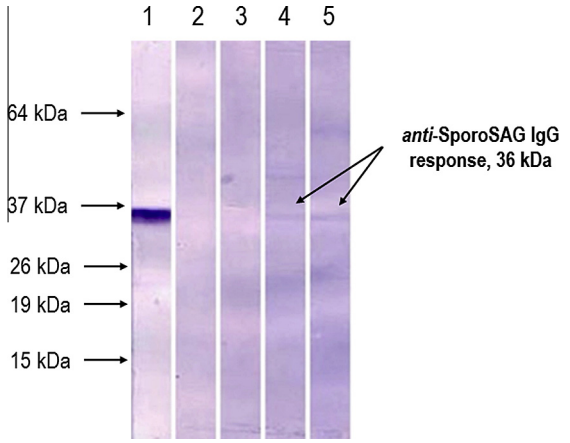

Fig. 3. Determination of *anti*-SporoSAG IgG antibody responses induced by vaccinations. Lane 2: Probe of rSporoSAG protein using pooled sera of mice vaccinated with empty pEGFP (lane 2), pBcl-xL (lane 3), pSporoSAG (lane 4), pSporoSAG/Bcl-xL (lane 5). Purified rSporoSAG has been probed with *anti*-poly histidine antibody (lane 1).

excreting IFN-γ in pBcl-xL group was significantly higher than pSporoSAG vaccinated control group ($P = 0.0001$).

$CD4^+$ T lymphocytes excreting IL-4 ratio of all vaccination groups were significantly higher than empty pEGFP control ($P < 0.0001$; designated by "***" in Fig. 4C). The level of $CD4^+$ T lymphocytes excreting IL-4 was in pBcl-xL group was significantly higher than pSporoSAG vaccinated control group ($P < 0.01$). The ratio of $CD8^+$ T lymphocytes excreting IFN-γ in pSporoSAG/Bcl-xL vaccinated group was significantly higher than pBcl-xL control group ($P < 0.05$). pSporoSAG/Bcl-xL vaccinated group as significantly higher than pSporoSAG vaccinated control group ($P = 0.0004$).

Overall, the pSporoSAG/Bcl-xL induced strong IFN-γ secreting $CD8^+$ T lymphocyte response which is protective against *T. gondii*. In addition, pSporoSAG/Bcl-xL induced strong IL-4 secreting $CD4^+$ response indicative of Th1/Th2 balanced response. $CD3^+$ cells from all experimental and control groups proliferated to comparable levels in response to concanavalin A.

Discussion

Since 1990s, several vaccine candidate antigens have been tested mainly using DNA vaccine formulations against mostly the tachyzoite form of the parasite. The antigens used in these studies are from SRS family proteins (SAG1, SAG2, SAG3, and SAG2CDX) [18–21], micronemal proteins (MIC1, MIC2, MIC3, MIC4, MIC6, MIC8, MIC11, and MIC13) [22–28], dense granule proteins (GRA1, GRA2, GRA4, GRA6, and GRA7) [16,29–32], rhoptry proteins (ROP1, ROP2, ROP4, ROP5, ROP7, ROP8, ROP16, and ROP18 [31,33–39], and other proteins such as RON4 [40], M2AP and AMA1 [23], serin protease inhibitor-1 [41], immune mapped protein-1 [42], HSP70 and HSP30 [43], MAG1 [44], eukaryotic initiation factor-2α [45], eukaryotic translation initiation factor 4A [46], cyclophilin [47], calcium-dependent protein kinase 3 [48], protein disulfide isomerase [49], aspartic protease 1 and cathepsin proteases [50,51], perforin-like protein 1 [52], rhomboid protein 1 [53].

Protective immunity against *T. gondii* is achieved by Th1 responses, especially through IFN-γ secreting $CD8^+$ T lymphocytes [2,13]. Therefore a vaccine against toxoplasmosis requires using MHC-I restricted antigens to induce $CD8^+$ T lymphocytes response. Moreover a vaccine against toxoplasmosis is required to induce protective immune response consecutively to all (bradyzoite/sporozoite and tachyzoite) forms of the parasite since the tachyzoite has the ability to invade the host cell abruptly and immediately embeds itself in a protective parasitophorous vacuole which keeps away the host immune response. Furthermore, in the natural route of infection, only bradyzoites and sporozoites take place.

Oocysts are shed by infective felids to environment and sporozoites form inside the oocyt at the end of sporulation process which takes several days outside. Upon ingestion by the intermediate host, the sporozoites are released inside the distal intestine ad

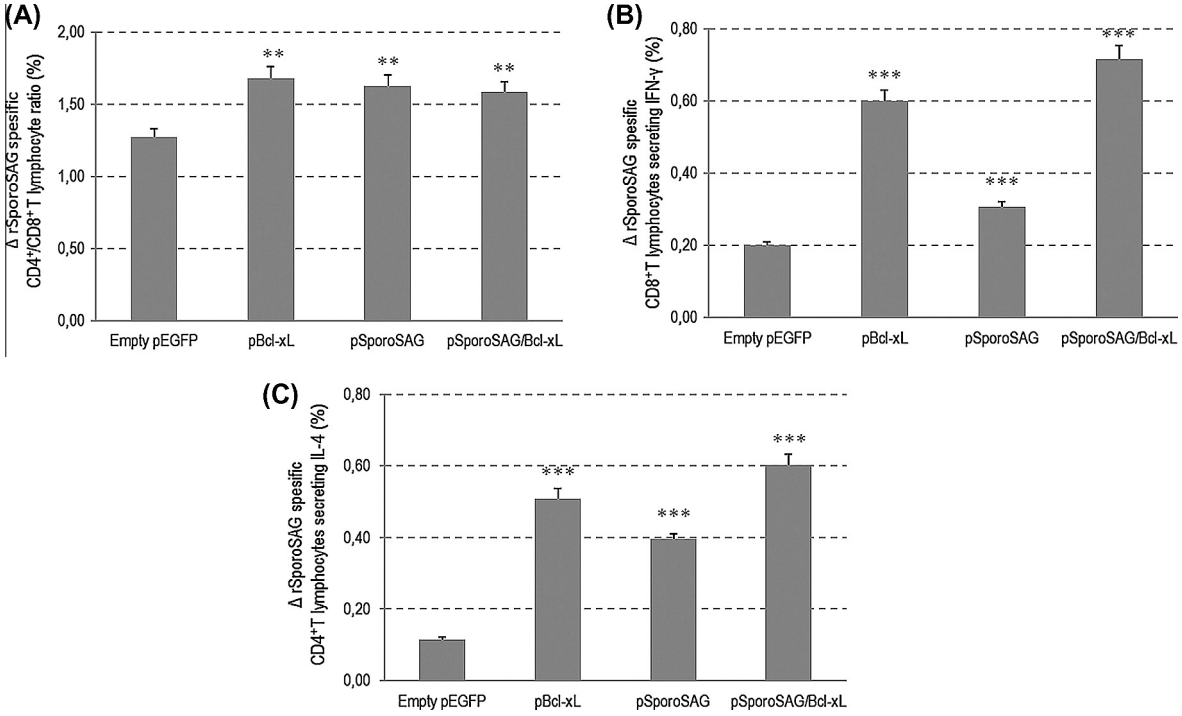

Fig. 4. Determination of *anti*-SporoSAG specific T lymphocyte response after vaccination using Western Blotting. (A) rSporoSAG protein specific $CD4^+$ and $CD8^+$ T lymphocyte ratio; (B) rSporoSAG protein specific $CD8^+$ T lymphocytes secreting IFN-γ; (C) rSporoSAG protein specific $CD4^+$ T lymphocytes secreting IL-4.

host cell invasion initiates. The mechanism of sporozoite for host cell invasion has not been clearly identified yet. However a two-step process has been defined for sporozoites wherein a primary widened vacuole forms initially and then the sporozoite proceed to form sophisticated and tighter secondary vacuole in which it grows [54].

SporoSAG (TGME49_058550) is from the SRS family proteins of *T. gondii* that is the most abundantly expressed protein on the infectious sporozoite surface as shown by transcriptomic and proteomic analysis conducted on oocysts/sporozoites [55,56]. SporoSAG protein has attachment function for host cell invasion [14,57]. The protective immune response stimulating efficacy of more than 40 antigens have been determined against toxoplasmosis in previous studies however the most abundantly expressed surface protein of sporozoite has not been evaluated yet. To demonstrate the immune stimulating properties of SporoSAG protein, a group of Swiss mice were experimentally infected with 8–10 fresh sporulated oocysts (containing sporozoites) perorally and *anti*-SporoSAG IgM and IgG antibodies were detected using a recombinant ELISA by our study group (unpublished data).

Therefore, SporoSAG was selected as DNA vaccine candidate antigen in this study due to its antigenic properties and role in host cell invasion. Thereafter, a dual promoter DNA vaccine containing SporoSAG and *anti*-apoptotic Bcl-xL gene was generated to increase the antigen specific $CD8^+$ T lymphocyte response. To induce stronger immune responses by DNA vaccination, using molecular adjuvants is a powerful approach. These molecular adjuvants can be any immunogenic protein or any cytokine, chemokine depending on the desired immune response to be elicited [58]. Bcl-xL is an antiapoptotic Bcl-2 family member which is localized on the outer membrane of mitochondria. There, it inhibits Bax (Bcl-2 associated X protein; mostly found in cytosol and upon apoptosis it is particularly associated to mitochondrial membrane forming large oligomers that permeabilize the outer mitochondrial membrane) induced pores, preventing the release of cytochrome c into cytoplasm to induce caspase dependent cell death [59,60]. In addition, Bcl-xL protein was shown to increase the antigen specific $CD8^+$ T lymphocyte response in different DNA vaccination studies [15,17]. *In vitro* study of a DNA vaccine encoding Bcl-xL against FMD (Foot-and-mouth disease) showed that Bcl-xL significantly prolonged the survival of transfected cells ($P < 0.001$) [15]. In another study, DNA vaccines encoding Bcl-xL and a part of human papillomavirus type 16 (HPV-16) E7 proteins were administered together and it has been showed that DNA vaccine encoding Bcl-xL prolonged the E7-specific $CD8^+$ T-cell responses [17]. Similarly, a DNA vaccine encoding Single chain trimer (composed of the most immunogenic epitope of human papillomavirus type 16 E6 protein, beta2-microglobulin, and MHC class I heavy chain) DNA vaccine combined with Bcl-xL increased E6-specific $CD8^+$ T lymphocyte immune response [61]. In the present study, *in vitro* cell survival of pSporoSAG and pSporoSAG/Bcl-xL transfected cells were significantly higher than empty EGFP control plasmid ($P < 0.05$). Moreover, the percentage of pSporoSAG/Bcl-xL plasmid transfected cells was higher compared to pBcl-xL plasmid (Fig. 1B). Western blotting showed the accurate expression of Bcl-xL *anti*-apoptotic protein in Bcl-xL expressing plasmids (Fig. 2). Altogether, Western Blotting and flow cytometer analyses rationalized Bcl-xL expression that impedes apoptotic cell death.

The ability of sporozoite surface protein "SporoSAG", to induce protective immune response using a DNA vaccine has been evaluated for the first time in this study. According to the results, the mice groups vaccinated with pSporoSAG/Bcl-xL DNA vaccine as well as pSporoSAG control vaccine induced strong IgG responses against recombinant SporoSAG protein (Fig. 3; lanes 4 and 5). A protective DNA vaccine against toxoplasmosis has to induce IFN-γ secretion from $CD8^+$ T lymphocytes. In the present study, the ratio of $CD8^+$ T lymphocytes excreting IFN-γ in pSporoSAG/Bcl-xL vaccinated group was significantly higher compared to pSporoSAG ($P < 0.0001$) and pBcl-xL ($P < 0.05$) control groups. In addition, the ratio of pSporoSAG control group was significantly higher compared to control group mice vaccinated with empty pEGFP ($P = 0.0005$). The results of IFN-γ secreting $CD8^+$ T lymphocytes reveal the ability of SporoSAG protein to induce protective immune response against toxoplasmosis. The higher ratios of $CD8^+$ T lymphocytes excreting IFN-γ in pSporoSAG/Bcl-xL and pBcl-xL groups compared to pSporoSAG vaccinated group show the low $CD8^+$ T lymphocyte stimulating efficiency of SporoSAG protein when used alone (Fig. 4B). In addition, pSporoSAG/Bcl-xL as well as pSporoSAG induced strong IL-4 secreting $CD4^+$ response indicative of Th2 polarized response (Fig. 4C). Altogether the pSporoSAG/Bcl-xL seems to induce strong Th1 and Th2 responses at the same time slightly biasing towards Th1 response which is an indication of protection against toxoplasmosis.

Overall, an ideal vaccine against toxoplasmosis should sequentially block the bradyzoites/sporozoites during the initial phase of the infection as well as tachyzoites after stage conversion. Therefore, a vaccine that protects from all forms of the *T. gondii* is required. This aim can be achieved by using a highly antigenic protein expressed by all forms of the parasite or combining antigenic proteins expressed by each form of the parasite in a multiprotein vaccine formulation. The results of this study show that SporoSAG, the most abundantly expressed protein on the surface of the sporozoites, can be included to multivalant vaccine formulations in future studies to increase the protective efficacy of vaccines aiming to protect infections acquired through ingestion of *T. gondii* oocysts.

Acknowledgements

This study was supported partly by the Grants given by The Scientific and Technological Research Council of Turkey (107S359) and the Ege University Science and Technology Center, Turkey (2008BIL008) to Y.G. The authors would like to acknowledge Michael White, Ph.D. for providing the backbone of SporoSAG gene. The topic of the study received a research award from the Ege University, Science and Technology Center and presented orally in 20th ECCMID (European Congress of Clinical Microbiology and Infectious Diseases) in Vienna, Austria, 2010. The authors state that they have no conflict of interest.

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